Kidney International, Vol. 59 (2001), pp. 2084–2094

Cell surface heparan sulfate proteoglycans control the response of renal interstitial fibroblasts to fibroblast growth factor-2

Aled Clayton, Janet Thomas, Gareth J. Thomas, Malcolm Davies, and Robert Steadman

Institute of Nephrology, University of Wales College of Medicine, Cardiff, Wales, United Kingdom

Cell surface heparan sulfate proteoglycans control the response of renal interstitial fibroblasts to fibroblast growth factor-2.

Background. While the progression of renal disease to end stage is strongly correlated with tubulointerstitial changes, the control of the fibrotic process within the interstitium is poorly understood. Basic fibroblast growth factor (FGF-2) has been implicated as a major growth factor involved in fibroblast activation and extracellular matrix synthesis. Furthermore, in many cells, the activity of FGF-2 is controlled by a low-affinity but high-capacity interaction with heparan sulfate (HS) proteoglycans (PGs), such as members of the syndecan family. These molecules are likely to be central to the control of interstitial fibrosis, but as yet, there has been no characterization of their synthesis by interstitial cells.

Methods. The expression of HSPG on the surface of NRK 49F fibroblasts was demonstrated by immunohistochemistry and by metabolic labeling with [^{35}S]-sulfate. HSs were characterized by specific enzymatic digestion, size exclusion chromatography, and anion exchange chromatography. The mRNA for syndecan 1 through syndecan 4 in the fibroblasts was detected by semiquantitative reverse transcription-polymerase chain reaction. Fibroblast proliferation was measured by the MTT assay.

Results. Immunohistochemistry and [^{35}S]-sulfate-labeling demonstrated that renal fibroblasts expressed HSPGs on their surface. Furthermore, enzymatic removal of these HS (but not chondroitin sulfate) glycosaminoglycan (GAG) chains, or inhibition of GAG sulfation, abolished the proliferative response of both NRK cells and primary human cortical fibroblasts to FGF-2 but not to platelet-derived growth factor. The addition of conditioned medium, containing HS-GAG fragments, restored the proliferative response to FGF-2, confirming the specificity of the interaction. Finally, the mRNA for all four syndecans was detected in the fibroblasts, and that for syndecan 1 in particular was up-regulated by FGF-2.

Conclusions. The present study demonstrates that the expression of cell surface HSPG was essential for the proliferation of renal fibroblasts in response to FGF-2, and therefore may play a major role in the development and persistence of a proliferating phenotype during interstitial nephritis.

Key words: fibrosis, interstitial inflammation, syndecan family, cell proliferation, progressive renal disease, extracellular matrix, prosteoglycans.

Received for publication June 30, 2000
and in revised form November 21, 2000
Accepted for publication December 26, 2000

Although glomerular and interstitial inflammation are hallmarks of progressive renal disease [1], the events determining whether the initial insult progresses to end-stage renal failure are poorly understood. What is clear, however, is that in many instances, the decline in renal function is more closely linked to interstitial extracellular matrix accumulation and expansion than to changes in the glomerulus [2, 3].

Fibrosis in other organs is considered the direct result of fibroblast activation, and there is a well-described sequence of matrix protein deposition that occurs during fibrotic progression. In the cortical interstitium of the kidney, fibroblasts are thought to play a major role in matrix deposition and hence represent an important cell, dictating whether or not the insulted kidney proceeds to renal failure [4–11]. The synthesis, deposition, and turnover of matrix components by fibroblasts are controlled by soluble mediators and growth factors such as transforming growth factor-β (TGF-β) and basic fibroblast growth factor (FGF-2). Furthermore, in many cells, the biological effect of these mediators is modulated by their interaction with matrix- and cell-associated proteoglycans (PGs) [12–15].

Proteoglycans are specialized glycoproteins with a protein core, which is covalently substituted with a complex pattern of glycosaminoglycan (GAG) side chains. These GAG chains confer a strong anionic charge, and in the kidney their principal role is to provide the charge barrier of the glomerular basement membrane, which is essential for the efficient control of the macromolecular permeability of the membrane. Perturbation of this an-

ionic barrier, which is principally provided by the heparan sulfate (HS) PGs agrin and perlecan, leads to a loss of filter function and to proteinuria [16–19]. Other HSPGs, such as glypican, CD44, and members of the syndecan family of transmembrane PGs [20–24], are expressed on the cell surface and, in some cells, serve as low-affinity binding sites for several different heparin-binding growth factors, including FGF-2 [25]. The binding of growth factors to the GAG component of these molecules is a prerequisite for the increased local concentration of the factor, its stability, conformation, and subsequent transfer to specific, high-affinity signaling receptors [24].

The clinical significance of the expression of particular HS-GAG structures was demonstrated by Morita et al, who examined the expression of HS-GAG chains in renal biopsy sections [26]. While total HS expression was increased in both the glomerulus and the tubulointerstitium, FGF-2 binding was only increased on cells in the interstitium, suggesting that the expression of specific cell surface HSPG may play a major role in controlling fibrotic events in the interstitium. This again emphasizes the potential importance of the interstitial fibroblast in fibrotic progression. Despite great interest in recent years in characterizing the renal interstitial fibroblast and understanding its function, however, the metabolism and turnover of growth factor-binding PGs by this cell have not been examined. The present report examines the expression of cell surface HSPGs by renal interstitial fibroblasts and addresses their importance for the response of the cell to FGF-2.

METHODS

Cell culture

Renal fibroblasts (NRK49F) were purchased from the European Collection of Animal Cell Cultures (ECACC Number 86101301; Center for Applied Microbiology & Research, Salisbury, Wiltshire, UK). Human cortical fibroblasts were prepared from normal tissue obtained at nephrectomy as described previously [8]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 containing, 2 mmol/L L-glutamine, 100 U/mL of penicillin, and streptomycin, and they were supplemented with 5% fetal calf serum (FCS; Hyclone, Erembodegem-Aalst, Belgium) at 37°C in a humidified incubator in an atmosphere of 5% CO_2. All tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Paisley, UK).

Estimation of cell numbers

Both direct cell counting and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay were used to determine renal fibroblast cell numbers [27]. The cells were seeded at 5×10^3 cells/well in 96-well plates and cultured in 5% FCS for 24 hours to subconfluence. Cells were then incubated under the appropriate experimental conditions. At the end of each incubation, the medium was removed, and for analysis by MTT assay, 100 μL of MTT (Sigma, Poole, UK; 1.25 mg/mL in DMEM/Ham's medium) were added to the monolayers. The cells were incubated at 37°C for four hours. The formazan precipitate formed was solubilized by incubation with 20% sodium dodecyl sulfate (SDS) and 50% dimethyl formamide overnight, and the absorbance was determined at 600 nm. The lower limit of sensitivity of the MTT assay was established as equivalent to 880 ± 79 cells with a correlation coefficient of 0.95 between cell number and absorbance at 600 nm. For cell counting, at the end of each incubation, the medium was removed, and cells were released from each well by incubation with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) in calcium- and magnesium-free phosphate-buffered saline (PBS), pH 7.3. Cells were then counted in a hemocytometer.

The conditions needed to maintain the fibroblasts in stable growth-arrested conditions were established by seeding cells onto 96-well plates and incubating them in 5% FCS for 24 hours. The cells were then exposed to fresh medium containing either 0 to 5% FCS or 0 to 1% lactalbumin hydrolysate (LH) for up to 72 hours, and the cell numbers were estimated at 24-hour intervals by MTT assay. Serum concentrations of 1% FCS or higher were proliferative, while at concentrations of 0.1% or below, cells lost viability. At 0.5% FCS, or at 0.2% LH, the cells became quiescent and remained viable throughout the culture periods tested up to 120 hours (data not shown).

In all subsequent experiments to evaluate fibroblast proliferation, cells were growth arrested for 72 hours before treatment or stimulation. In proliferation experiments, quiescent fibroblasts were incubated either in serum-free medium, supplemented with 0.2% lactalbumin (negative control), 5% FCS (positive control), or serum-free medium containing FGF-2 or platelet-derived growth factor (PDGF)-BB (R&D Systems Europe Ltd., Abingdon, Oxford, UK).

Metabolic labeling of proteoglycans

We have previously described the metabolic labeling of renal cells with [^{35}S]-sulfate [28, 29] and in the current report this was the same with the exception that the medium was based on DMEM/Ham's F12. At confluence, the growth medium was removed. The cells washed three times with PBS, and the cells were growth arrested by maintenance in 0.2% LH in DMEM/Ham's-F12 for 48 hours. The cells were washed with PBS and metabolically labeled with 50 μCi/mL carrier-free [^{35}S]-sulfate in DMEM/Ham's-F12, containing 10% of the normal sulfate concentration and supplemented with either 5%

sulfate-depleted FCS (prepared by exhaustive dialysis against low sulfate DMEM-Ham's F12) or 0.2% lactalbumin for 24 hours, to attain a steady-state equilibrium between synthesis and the combined pathways of secretion and digestion.

Analysis of cell surface proteoglycans

After labeling with [^{35}S]-sulfate, the culture medium was collected. The cells were washed three times with PBS, and the cell surface PGs were released by incubation with 20 μg/mL trypsin in PBS on ice for five minutes. This treatment has been shown previously to release cell surface PGs without affecting cell viability [30, 31]. The trypsin-inaccessible material remaining in the intact cell layer was extracted with 4 mol/L urea in Tris-Bis buffer, pH 6.0, containing 0.1% CHAPS. The trypsin-released and trypsin-resistant materials were then separately passed over a column of DEAE Sephacel, equilibrated with 4 mol/L urea in Tris-Bis buffer, pH 6.0, containing 0.1% CHAPS [28, 29]. The column was washed with 0.3 mol/L NaCl in the urea buffer, and the bound [^{35}S]-labeled PGs were eluted with 1.5 NaCl in urea buffer. These were then dialyzed against 50 mmol/L Tris HCl, pH 8.0, containing 50 mmol/L NaCl and incubated with 125 mU of chondroitin ABC lyase at 37°C. The remaining [^{35}S]-labeled HSPGs were then precipitated with three volumes of 1% wt/vol potassium acetate in 95% ethanol in the presence of 50 μg/mL each of heparin and chondroitin sulfate as coprecipitants and analyzed on Sepharose CL-4B columns equilibrated with 4 mol/L guanidine in 50 mmol/L sodium acetate buffer, pH 6.0, containing 0.5% Triton X-100 and 0.05% sodium azide.

Immunohistochemical identification of HS-GAG chains

Fibroblasts were fixed and permeabilized in cold 50% acetone:50% methanol (−20°C) for five minutes followed by three washes in PBS. Nonspecific sites on the fixed cells were blocked with 1% bovine serum albumin (BSA) and 1% dried milk in PBS, and the slides were incubated with an anti-HSPG antibody that specifically recognizes sulfated HS-GAGs (10E4-mAb, 1 in 30 dilution; AMS Biotechnology, Whitney, UK) at room temperature for one hour. The cells were washed four times with 1% BSA, 1% dried milk in PBS and then incubated with the secondary antibody FITC-conjugated anti-IgM (1:80; Sigma) at room temperature for one hour. The cells were washed with PBS, mounted in Vectashield fluorescent mountant (Vector Laboratories, Peterborough, UK), and examined on a Leica Dialux 20 fluorescent microscope.

Enzymatic removal of HS-GAG chains

Fibroblasts were grown to subconfluence and incubated with either a cocktail of 0.3 mU each of heparitinases I, II, and III (Grampian Enzymes, Orkney, UK) or chondroitin ABC lyase (50 mU; ICN, Basingstoke, UK) as a control for up to 48 hours. The cells were then incubated either in serum-free medium, supplemented with 0.2% lactalbumin (negative control), 5% FCS (positive control), or in serum-free medium containing FGF-2 or PDGF-BB for the appropriate times. The fibroblasts were then washed in PBS and fixed for immunocytochemistry, or cell numbers were estimated by MTT assay, as described previously in this article.

Inhibition of HSPG sulfation

Sodium chlorate was used to inhibit sulfate adenyltransferase activity and thus prevent sulfate donation to newly synthesized GAG chains [32]. Renal fibroblasts were growth arrested for 72 hours in sulfate-free DMEM (GIBCO-BRL) containing 0.5% sulfate-depleted FCS. The medium was then removed, and the cells were incubated in fresh sulfate-free DMEM and 0.5% sulfate-depleted FCS, containing sodium chlorate (0 to 30 mmol/L) for 48 hours. The absence of sulfated cell surface GAG was confirmed by immunohistochemical staining with antibody 10E4 as described previously in this article. Fibroblasts were then incubated in serum-free medium, supplemented with 0.2% lactalbumin, 5% FCS, or in serum-free medium containing FGF-2, and cell numbers were estimated by MTT assay as described previously in this article.

Semiquantitative RT-PCR analysis of fibroblast syndecans

In all experiments to isolate and identify the mRNA for specific proteins, fibroblast monolayers were established and incubated in six-well plates. At the end of the appropriate incubation, the cells were washed once in PBS, pH 7.3, and then lyzed by the addition of 1 mL/well of RNA-isolator (Genosys, Cambridge, UK). Chloroform (0.1 mL) was then added, and the mixture was shaken for 30 seconds. Samples were incubated at 4°C for 5 minutes and centrifuged at 20,000 × g for 15 minutes. The aqueous phase was then decanted and mixed with an equal volume of isopropanol. This was incubated at −70°C for 24 hours, and the precipitate was pelleted by centrifugation at 20,000 × *g* at 4°C for 20 minutes. The isolated RNA was washed twice with 500 μL of 75% ethanol, and the pellet was air dried at room temperature for 5 to 10 minutes. The RNA was dissolved in 10 μL of H_2O, and its concentration was calculated from the absorbance at 260 nm. The integrity of the RNA was assessed by flat-bed electrophoresis.

Reverse transcription (RT) was performed using the random hexamer method. RNA 1 μg was added to 1 μL of 100 mmol/L random hexamers (Pharmacia Biosystems, Milton Keynes, UK), 5 μL NTP (2500 nmol mixed nucleotides: dATP, dCTP, dGTP, dTTP; GIBCO/BRL),

Table 1. Syndecan polymerase chain reaction (PCR) primer constructs

Gene	Primer pairs	Size
Syndecan-1	3′-TCCAGAACGGGTGGAACTGG-5′ 5′-GCAGTCTTAGTGCAACCCGG-3′ or 3′-GGAGGCACTTCTGTCATCAA-5′ 5′-AGCACTTCCTTCATGTCCAA-3′	339 bp[a] 212 bp[b]
Syndecan-2	3′-CTCTGGCTCAGGAGATTATG-5′ 5′-CACCAACAACAGGATGAGGA-3′	345 bp[b]
Syndecan-3	3′-GATGAGCCAGAGGTGCAAGT-5′ 5′-GCCACCTACGATCACAGCTA-3′	259 bp[b]
Syndecan-4	3′-AGGCGAGTCGATTCGAGAGA-5′ 5′-TCAGAGCTGCCAAGACCTCA-3′	394 bp[b]
α-Actin	3′-GGAGCAATGATCTTGATCTT-5′ 5′-TCCTGAGGTACGGGTCCTTCC-3′	204 bp[a]

[a] Primers designed in house using Lazergen™ primer design software
[b] Primer sequences obtained from published reports [33, 34]

2 μL 1 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl (15 mmol/L $MgCl_2$, and 0.001% wt/vol gelatin) and 2 μL of 100 mmol/L dithiothreitol. The solution was heated to 95°C for five minutes followed by 4°C for two minutes. One microliter Ribonuclease inhibitor (RNAsin; Promega, Southampton, UK) and 1 μL of Maloney monkey leukemia virus (M-MLV™) Superscript (RNase H-reverse transcriptase; GIBCO/BRL) were added to each sample and incubated at 22°C for 10 minutes, 42°C for 60 minutes, and then 95°C for five-minute cycles on a Perkin Elmer Cetus DNA thermal cycler 480. The cDNA products were stored at −20°C.

The polymerase chain reaction (PCR) was carried out in 50 μL (final volume) of 10 mmol/L Tris-HCl buffer, pH 8.3, containing 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 mmol/L dNTP, 0.001% wt/vol gelatin, 0.5 mmol/L of each primer for the product of interest, and 1.25 U of AmpliTaq-Gold DNA polymerase (Applied Biosystems, Warrington, UK). The primers were designed from the published cDNA sequences [33, 34], using Lasergene Primer Select software (Appligene, Chester-le Street, UK; Table 1). Mineral oil (50 μL) was added to each tube, and thermal cycling was carried out as shown in Table 2. Each product was amplified in separate tubes, and 10 μL were then separated on 3% agarose gels (2% Nusieve GTG agarose; Flowgen, Sittingbourne, UK; and 1% Ultrapure agarose; GIBCO BRL), containing 0.5 mg/mL ethidium bromide at 70 V for one hour. The products in the gels were visualized and photographed under ultraviolet light. Scanning densitometry using a BioRad GS 670 allowed the ratio of each product to that of the actin housekeeping gene to be calculated. The values were then normalized to the level of the unstimulated control (for dose response experiments) or the zero time control (for time course experiments).

Table 2. Amplification cycles

Taq activation	22–34 cycles of	Followed by
	94°C for 0.6 minutes	
95°C for 10 minutes	60°C for 0.6 minutes	94°C for 0.6 minutes
72°C for 5 minutes	72°C for 1 minute	60°C for 10 minutes

Statistical analysis

Results are expressed as means ± SEM of N experiments. The two-tailed paired Student t test was used to compare values with controls. A value of $P < 0.05$ was considered significant.

RESULTS

Renal fibroblasts express cell-surface HSPG

To demonstrate the expression of HSPG on the surface of renal fibroblasts, cells were metabolically labeled with [^{35}S]-sulfate for 24 hours, followed by a brief incubation with trypsin to release cell surface PGs. This treatment released the radiolabeled macromolecules from the cell surface without causing detachment of the cells. The radiolabeled molecules that remained associated with the cells (trypsin-inaccessible) were then extracted as described. Both batches of labeled material were then applied separately to DEAE-Sephacel to bind the PGs and allow the removal of other labeled proteins, and both were characterized (following removal of chondroitin/dermatan sulfates by digestion with chondroitin ABC lyase) on a Sepharose CL-4B column. Analysis of the trypsin-released material indicated a single peak of radioactivity, which eluted with a K_{av} of 0.45 (Fig. 1A). Further incubation of the labeled material with papain caused an increase in K_{av} to >0.6 (data not shown), indicating that the trypsin-released material was a PG and not free GAG chains. The analysis of the trypsin-inaccessible material indicated a broad distribution of molecules indicative of both intact HSPG (K_{av} 0.2 to 0.4) and HS-GAG fragments (K_{av} 0.7; Fig. 1B). These results indicate that the renal fibroblasts express HSPG on the cell surface and that there is also a population of intracellular labeled HS-GAGs, which may represent either newly synthesized HSPG or HS chains undergoing degradation.

Further confirmation of the presence of cell surface HSPG was obtained by incubating [^{35}S]-sulfate-labeled cells with heparitinase. The released material was purified on DEAE-Sephacel as described previously in this article and analyzed on Sepharose CL-4B. Since heparitinase specifically digests HS-GAG chains, the broad elution profile obtained represented HS fragments (K_{av} 0.5 to 0.8) derived from cell surface HSPGs that were not fully degraded to their constituent disaccharides (Fig. 2).

Finally, the presence of HSPG on the cell surface was

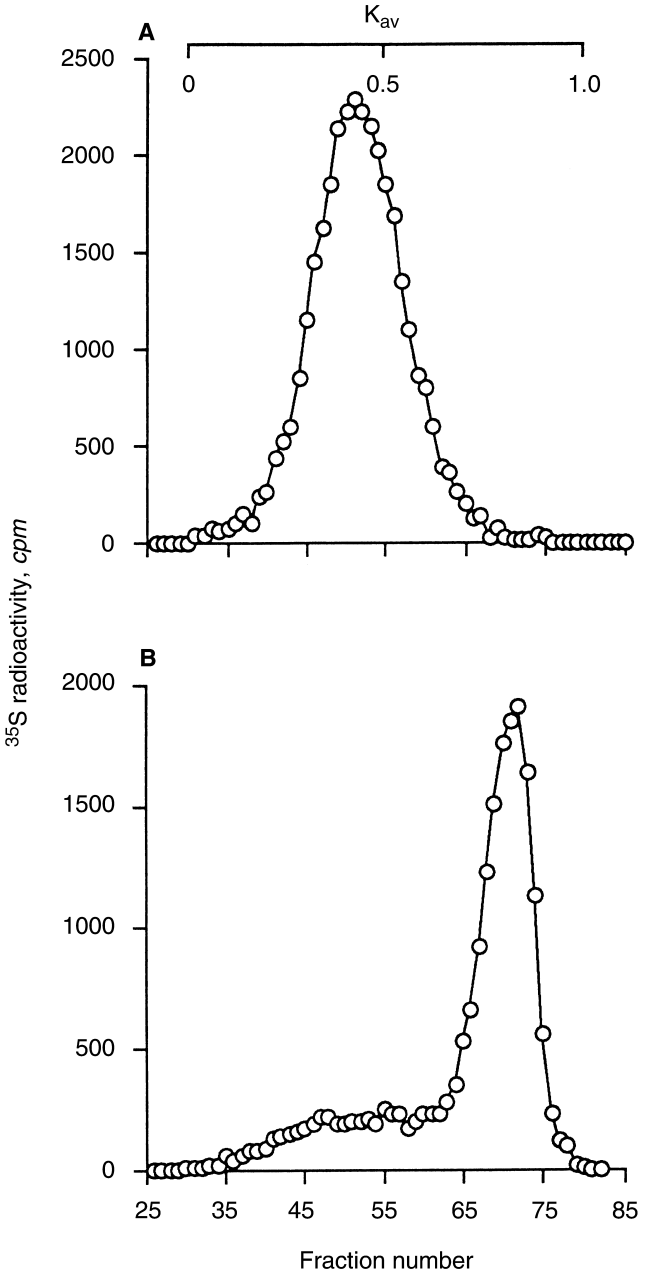


Fig. 1. Sepharose CL-4B chromatography of cell surface heparan sulfate proteoglycans (HSPGs). NRK fibroblasts were metabolically labeled with [^{35}S]-sulfate for 24 hours. The medium was removed, and the cell layer was incubated with 20 μg/mL trypsin on ice for five minutes. Portions of (*A*) the trypsin-released or (*B*) the trypsin-inaccessible material were applied to DEAE-Sephacel. The bound [^{35}S]-labeled material was eluted, incubated with chondroitin ABC lyase to remove CS/DS chains, and alcohol precipitated. Following reconstitution in 4 mol/L guanidine buffer, the labeled HS material was applied to a column of Sepharose CL-4B. Results are representative of four separate experiments.

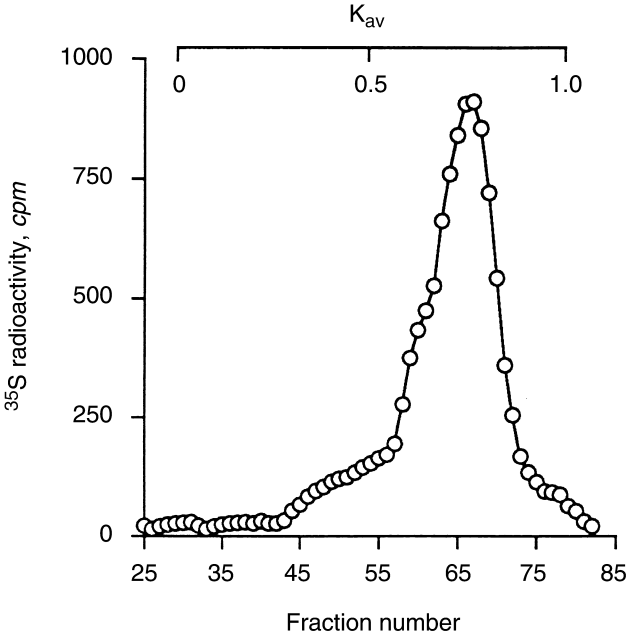


Fig. 2. Sepharose CL-4B chromatography of cell surface HS-GAG after limited heparitinase digestion. NRK fibroblasts were metabolically labeled with [^{35}S]-sulfate for 24 hours. The medium was removed. The cell layer was incubated for 30 minutes with a cocktail of heparitinases I, II, and III at 37°C, as described, and the released material was applied to a column of Sepharose CL-4B as in Figure 1. Results are representative of three separate experiments.

confirmed by immunostaining with monoclonal antibody 10E4 (Fig. 3a). This antibody recognizes intact, fully sulfated HS chains. Removal of immunoreactive HS epitopes by specific digestion with heparitinase prior to fixation and immunostaining completely removed immunoreactive HS epitopes from the cell surface (Fig. 3b). Perinuclear foci of anti-HS staining, however, were observed, suggesting that intracellular processing of HS was still occurring. Newly synthesized HS-GAGs were not detected at the cell surface during the course of the experiment or following extended incubations with heparitinase. In contrast, there was no effect of chondroitin ABC lyase on the detection of these HS epitopes (Fig. 3c). Neither heparitinase or chondroitin ABC lyase incubations affected the adhesive properties of the cells, their viability, or their proliferative response to FCS (data not shown).

Proliferation induced by FGF-2 requires cell surface HSPG

Having demonstrated the presence of cell surface HSPG, further experiments investigated whether they were involved in the proliferative response of renal fibroblasts to FGF-2. Initial experiments established the mitogenic potential of FGF-2 for the NRK and human cortical fibroblasts. Growth-arrested cells were cultured in 0.5 to 10 ng/mL FGF-2 for 48 hours at the end of which period cell numbers were determined by MTT assay or by direct cell counting. Fibroblasts were also incubated with FCS (0 to 5%) or PDGF-BB (0 to 50 ng/mL) as positive controls. Under these conditions, the fibroblast numbers increased in response to all three mitogens in a

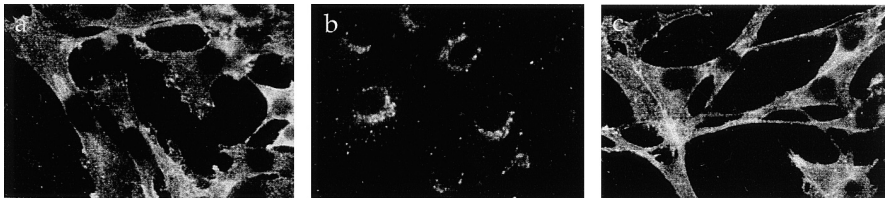


Fig. 3. Immunofluorescent staining of HS-GAG. NRK fibroblasts were grown to confluence in DMEM-Ham's F12 supplemented with 5% FCS. The medium was removed, and the cells were incubated with either (*a*) buffer alone, (*b*) heparitinase, or (*c*) chondroitin ABC lyase at 37°C for 30 minutes. The cells were washed free of enzyme, fixed, and stained for HS-GAG using antibody 10E4. Results are representative of three separate experiments. Magnification ×250.

dose-dependent manner (Fig. 4). In parallel experiments, cells were preincubated at 37°C for 30 minutes with heparitinase prior to incubation with the growth factors. This treatment of the cultures markedly and significantly inhibited the increase in cell numbers elicited by FGF-2 (Fig. 4A). In contrast, the removal of HS from the cell surface had no significant effect on proliferation induced by either PDGF-BB (Fig. 4B) or FCS (Fig. 4C). Thus, the inhibition of proliferation caused by removal of HS-GAGs was specific to the FGF-2 response. In further experiments, the response of primary human cells to FGF-2 was also shown to depend on the presence of cell surface HS-GAG chains (Table 3).

These results suggested that the expression of the HS-GAGs at the cell surface was essential for the response of the fibroblasts to FGF-2. To confirm that this was indeed the case, medium containing HS-GAG fragments, released by heparitinase treatment as described previously in this article (Fig. 2), was added to fibroblasts from which cell surface HS-GAG had been completely removed by heparitinase digestion. The cells were then incubated with 10 ng/mL FGF-2 for 24 hours. Under these conditions, the inclusion of the cell surface-derived HS-GAG fragments served to restore the responsiveness of the fibroblasts to FGF-2 in a concentration-dependent manner (Fig. 5).

Table 3. Effect of heparitinase and chlorate on the proliferation of human cortical fibroblasts

	Cell proliferation % *of control*		
	Untreated	Heparitinase treated	Chlorate treated
FGF-2 *ng/mL*			
0	100	100	100
0.1	118 ± 17	99 ± 18	104 ± 12
1.0	226 ± 9[b]	109 ± 34	110 ± 16
10.0	312 ± 12[b]	131 ± 19	121 ± 12
PDGF-BB *ng/mL*			
0	100	100	100
0.5	149 ± 12	118 ± 35	198 ± 24[a]
5.0	241 ± 28[b]	170 ± 20[a]	209 ± 17[b]
50.0	256 ± 29[b]	230 ± 34[b]	356 ± 38[b]
FCS %			
0	100	100	100
0.5	100 ± 8	99 ± 22	110 ± 10
1.0	201 ± 10[b]	224 ± 31[b]	167 ± 9[b]
5.0	260 ± 15[b]	245 ± 11[b]	245 ± 14[b]

Growth-arrested human cortical fibroblasts in 96-well plates were either treated with 0.3 U/mL each of heparitinase I, II, and III at 37°C for 30 minutes or were placed in sulfate-free medium containing sodium chlorate (30 mmol/L; detailed in the **Methods** section) or were left untreated. They were then incubated for 48 hours with either fetal calf serum (FCS), basic fibroblast growth factor (FGF-2) or platelet-derived growth factor (PDGF)-BB at the indicated concentrations. Cell numbers were determined by direct counting of cells released from the wells of 96-well plates. The results are expressed as the mean percentage ± SEM ($N = 3$) of cells present after 48 hours in serum-free medium.

Significant increases in cell numbers are shown as [a] $P < 0.05$, [b] $P < 0.01$.

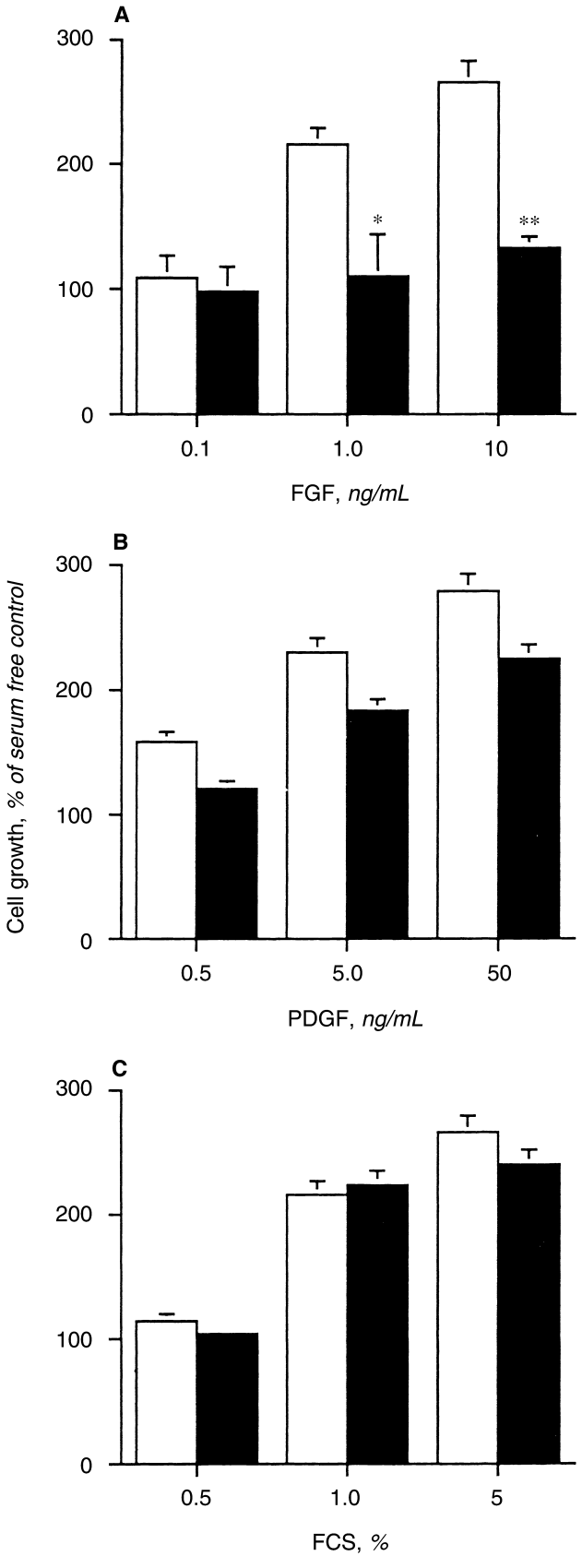
Proliferation induced by FGF-2 requires sulfation of the cell surface HSPG

3T3 fibroblasts maintained in sulfate-free medium and incubated with sodium chlorate synthesize GAG chains with reduced sulfation and lose their proliferative response to FGF-2 [35–37]. To confirm the role of HS-GAG chains and their sulfation in the interaction of the renal fibroblast with FGF-2, NRK 49F cells or human fibroblasts were grown in sulfate-depleted conditions in the presence of 30 mmol/L sodium chlorate. After culture for 24 hours, the cells were fixed and immunostained with antibody 10E4 as described previously in this article. Under conditions in which sulfate incorporation had been competitively inhibited by chlorate, there was no cell surface or intracellular staining, confirming the absence of sulfated HS-GAG chains (Fig. 6a). The inclusion of sodium sulfate, however, restored the staining to normal intensity, indicating that the inhibition was competitively reversible (Fig. 6b).

When cultured with FGF-2 under the same sulfate-depleted conditions for 48 hours, there was significant inhibition of the proliferative response to FGF-2 compared with cells in normal medium (Table 3 and Fig. 7). This effect was competitively and dose dependently reversed by the addition of sodium sulfate (Fig. 7). Thus, there is a requirement for intact, fully sulfated HS chains for the renal fibroblasts to undergo a proliferative response to FGF-2.

Renal fibroblasts transcribe all four syndecan genes

There are several cell surface HSPGs that could be expressed by the renal fibroblasts. Those most closely associated with an affect on FGF-2 interactions, however, are those of the syndecan family. RT-PCR was used to determine whether the cells expressed the mRNA for any of the four syndecans. Cells were grown to 50%



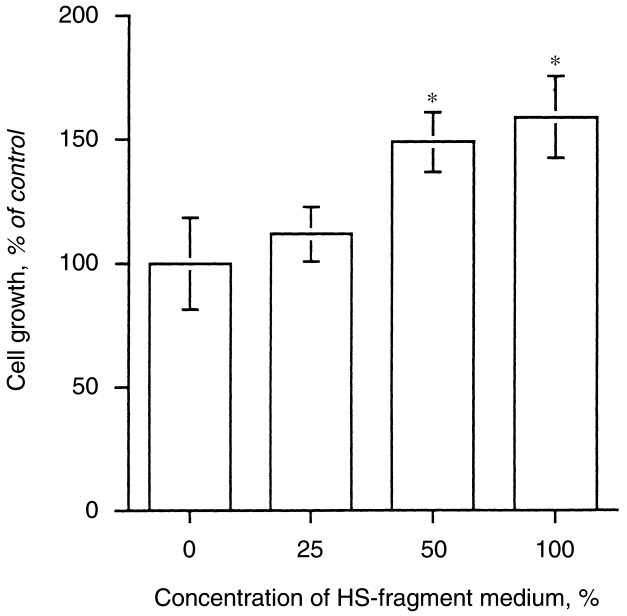


Fig. 5. HS-GAGs restore the response of NRK fibroblasts to FGF-2. Cells were grown to subconfluence. The medium was removed, and the cell layer was incubated with 0.3 U/mL heparitinase for 30 minutes. The supernatants were collected, bulked, and heated to 80°C for two hours to inactivate the heparitinases. The HS-GAG–depleted cells were then cultured with 10 ng/mL FGF-2 in the presence of a series of dilutions of the HS-GAG fragments in serum-free medium for 24 hours. Cell numbers were determined using the MTT assay. The results are expressed as the mean percentage ± SEM ($N = 4$) of the number of cells present after 24 hours in serum-free medium containing only 10 ng/mL FGF-2. *$P < 0.05$ compared with HS-GAG–depleted cells incubated with FGF-2 alone.

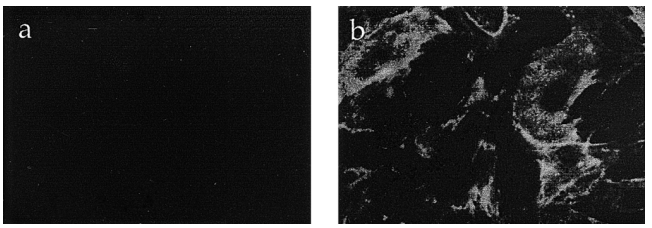


Fig. 6. Immunofluorescent staining of sulfate-depleted fibroblasts. Growth-arrested NRK fibroblasts were incubated in sulfate-free DMEM containing 0.5% sulfate-free FCS and (*a*) 30 mmol/L sodium chlorate or (*b*) 30 mmol/L sodium chlorate plus 10 mmol/L sodium sulfate for 48 hours. The cells were then fixed and stained for cell surface HS-GAG using antibody 10E4. Results are representative of three separate experiments. Magnification ×250.

Fig. 4. Cell surface HS-GAGs are required for FGF-2–mediated cell proliferation. Growth-arrested NRK fibroblasts were incubated in buffer alone (□) or with 0.3 U/mL each of heparitinases I, II, and III at 37°C for 30 minutes (■). The cells were then incubated in serum-free medium containing (*a*) FGF-2, (*b*) PDGF-BB, or (*c*) FCS in the presence or absence of fresh heparitinase for 48 hours. Proliferation was determined by MTT assay. Results are expressed as a mean percentage increase over that of cells incubated in serum-free medium alone ± SEM. *$P < 0.05$; **$P < 0.01$, $N = 3$.

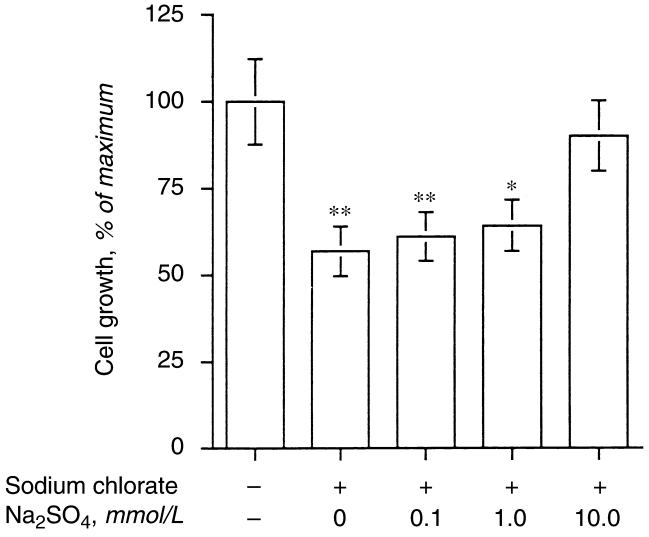


Fig. 7. Sulfate depletion inhibits the proliferation of renal fibroblasts in response to FGF-2. Growth-arrested NRK fibroblasts were incubated for 48 hours with 10 ng/mL FGF-2 in sulfate-free medium containing sodium chlorate (30 mmol/L) alone or with sodium sulfate (up to 10 mmol/L). Cell numbers were determined using the MTT assay. The results are expressed as the mean ± SEM ($N = 6$) of the percentage of the number of cells present after 48 hours in medium containing only FGF-2. *$P < 0.05$; **$P < 0.01$.

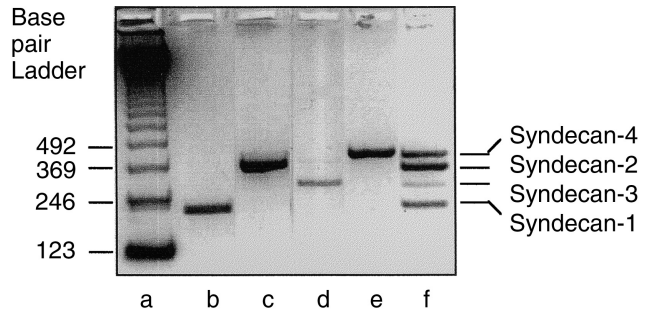


Fig. 8. Renal fibroblasts express syndecan mRNA. Total RNA was extracted from NRK 49F cells, and after RT, specific PCR was carried out as described. The individual products were electrophoresed in 3% agarose containing ethidium bromide and the gel photographed under ultraviolet light. Lane (a) base pair ladder markers, (b) syndecan 1, (c) syndecan 2, (d) syndecan 3, (e) syndecan 4, (f) a mixture of each primer product. The gel is representative of five separate experiments.

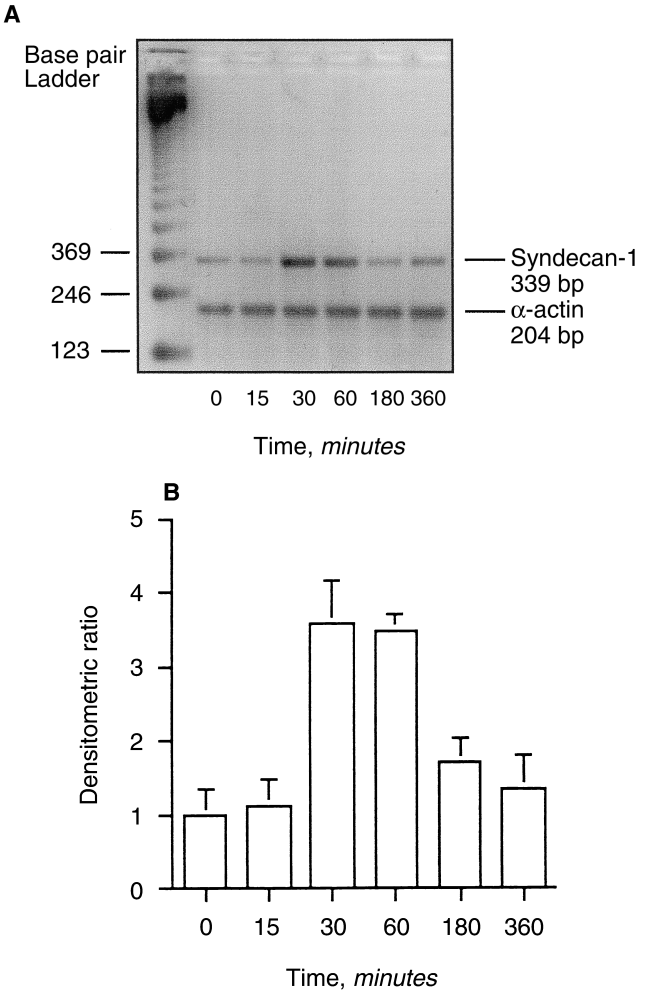


Fig. 9. Basic fibroblast growth factor (FGF-2) induces syndecan 1 mRNA. Growth-arrested NRK fibroblasts were cultured with DMEM-Ham's F-12 supplemented with 10 ng/mL of FGF-2. At the indicated times total RNA was extracted, and RT-PCR amplification was carried out. The products were electrophoresed in 3% agarose containing ethidium bromide, and the gel was photographed under ultraviolet light. α-Actin was used as the housekeeping gene. The gel shown is representative of three separate experiments. The densitometric ratio of syndecan to actin is shown for each time point normalized to the ratio at zero time (mean ± SEM, $N = 3$).

confluence in 5% FCS and growth arrested in 0.5% FCS for 72 hours. The total cell RNA was extracted, and RT-PCR was carried out using primers specific for syndecan 1, 2, 3, or 4 (Table 1). The PCR products were visualized on agarose gels. There was abundant expression of transcripts for syndecans 2 and 4, while syndecans 1 and 3 were expressed to a lesser extent (Fig. 8).

FGF-2 induces syndecan-1 mRNA

The possibility that FGF-2 may affect the expression of the HSPGs, which in turn control the response of the renal fibroblasts to FGF-2, was examined in a separate series of experiments. Growth-arrested fibroblasts were stimulated with 10 ng/mL FGF-2, and RNA was extracted at various times. RT-PCR demonstrated that exposure to FGF-2 triggered an immediate rise in syndecan 1 mRNA. This induction reached a maximum after 30 minutes and returned to basal levels by 6 hours (Fig. 9). During this period, there was little change in the mRNA for syndecan 2, 3, or 4 (data not shown).

DISCUSSION

This report describes the identification and expression of cell surface HSPGs on renal fibroblasts and demonstrates that they appear essential for the control of the

proliferative response of these cells to FGF-2. The presence of cell surface HSPGs was confirmed at several levels. First, they could be shown, following metabolic labeling with [^{35}S]-sulfate, to be accessible to both limited trypsin and limited heparitinase digestion. Second, the presence of immunoreactive HS-GAG on the cell surface was demonstrated by immunohistochemistry. Third, the cells were shown by RT-PCR to express the mRNA for each of the four syndecan cell surface HSPGs. In addition, the cell surface HSPGs were functional, and both their expression and their sulfation were essential for the initiation of a proliferative response to FGF-2. This was shown to be a mechanism that was specific for FGF-2 since neither proliferation in response to PDGF-BB nor to FCS was affected by removal of the cell surface HS chains.

Members of the FGF family serve diverse roles in cellular growth and differentiation during development, in healing, and in the pathogenesis of disease [38–40]. The cellular response to FGF is complex and in many cells is dependent not only on the expression of transmembrane tyrosine kinase receptors [12, 41], but also on the presence of HSPGs to facilitate FGF/receptor interaction. This is not the case, however, for all cells. For example, the rat thyroid cell line FRTL-5 uses a HS-independent mechanism for interaction with FGF-2 [42], while the proliferative effect of FGF-2 on other cells may be inhibited by HS chains [43]. Furthermore, not all cell-associated HS will bind FGF-2, and the expression of specific chain structures may vary at different stages of differentiation [44–46]. The renal fibroblasts used in our study, however, appear to be totally dependent on the presence of intact cell surface HS-GAG chains for the mediation of their response to FGF-2.

Studies by Rapraeger et al have demonstrated the restoration of the response of cells to FGF-2 following the addition of heparin [47]. This raised the possibility that native GAG chains derived from the surface of renal fibroblasts may also be able to perform this function. The importance of these chains was confirmed in experiments in which HS-GAG fragments, obtained by limited heparitinase digestion, were added to cells from which HS had been removed. Under these conditions, the inhibition of the proliferative response to FGF-2 was overcome. Thus, the endoglycosidase heparitinase had released HS fragments, which promoted the interaction of FGF-2 with its signaling receptors. Structural evidence has demonstrated that an FGF-heparin complex will mediate the dimerization of receptors, resulting in receptor activation and intracellular signaling. In an analogous manner, members of the cell surface HSPG syndecan family are also believed to bind FGF-2 and facilitate receptor dimerization and activation [48, 49]. It remains to be determined whether the configuration and propensity for dimerization of HS chains on the renal fibroblasts is similar to that described in these other systems.

The degree of sulfation was also a major factor controlling the response to FGF-2. This was shown by the use of sodium chlorate, a competitive inhibitor of the ATP-sulfate adenyltransferase. This enzyme participates in the formation of phosphoadenosine phosphosulfate, the high-energy sulfate donor required by the sulfotransferases, to add sulfate groups to the newly synthesized HS chains in the Golgi. At concentrations of approximately 30 mmol/L, the transferase is inhibited, and the HS chains at the cell surface bear nonsulfated GAG chains [32]. Renal fibroblasts remained viable at this dose of chlorate; however, they did not express sulfated HS chains at the cell surface, and their proliferative response to FGF-2 was significantly impaired. The addition of exogenous sulfate out competes chlorate, resulting in restoration of sulfation. This was sufficient to restore the proliferative effect of FGF-2 on the renal fibroblasts.

The role of FGF in renal disease (particularly FGF-2) has been extensively examined. As well as regulating the release of other profibrotic factors (such as TGF-β1) [50], FGF-2 modulates the proliferative activity of several cell types, contributing directly to renal injury and sclerosis [6, 21, 51–53]. Morita et al examined the expression of HSPG in biopsy sections of kidneys from patients with a variety of renal diseases [26]. Although HS-GAGs were observed on cells both in the glomerulus and the interstitium, the binding of FGF-2 was confined solely to regions of fibrotic injury in the interstitium. Whether this represents an alteration in the level of expression or a qualitative change in the structure of the GAG chains on cells in the interstitium is not clear.

Previous emphasis in the study of fibrogenesis has been on the identification of particular growth factors, such as FGF, involved in modulating fibroblast behavior. An investigation of the interaction between these growth factors, their receptors, and growth factor-binding molecules such as HSPGs is essential for increasing our understanding of fibrotic mechanisms. Alterations in the structure, charge, or size of HSPG molecules by fibroblasts in areas of fibrosis is a distinct possibility [26, 54], and such changes leading to increased growth factor binding may lead to an inappropriately enhanced response to growth factors. In culture, fibroblasts isolated from fibrotic kidneys and cultured in the presence of serum have been described as hyperproliferative compared with their nonfibrotic counterparts [55, 56]. An increased HSPG-mediated growth factor binding may explain the apparent hyperproliferative phenotype of fibroblasts derived from areas of fibrosis. Furthermore, the observation that FGF-2 induced the transcription of de novo syndecan 1 mRNA suggests that there may be a potent positive feedback loop that would be expected to amplify the fibrotic response. In this respect, the renal fibroblasts

behaved in a manner similar to that of 3T3 fibroblasts, in which syndecan 1 expression was stimulated by FGF-2 but not by epidermal growth factor [57]. Not all cells respond in this way, however, and in keratinocytes the syndecan 1 gene was stimulated by epidermal growth factor but not by FGF-2 [57].

Our data demonstrate a requirement for cell surface HSPG to mediate the biological response of renal fibroblasts to FGF-2. We have initially examined the expression of the four syndecan genes in this regard. Other cell surface molecules such as CD44 and glypican, however, are also substituted with HS GAG chains, and future studies will evaluate which of these receptors are involved in the interaction with FGF-2. The complex interplay therefore between profibrotic growth factors and HSPGs may be critical to our understanding of the molecular mechanisms of fibrotic renal disease. Furthermore, a more detailed understanding of the control of expression of cell surface PGs may be the key to the design of selective therapies for progressive renal disease.

ACKNOWLEDGMENTS

We would like to acknowledge the financial support of the National Kidney Research Fund, the Welsh Scheme for the Development of Health and Social Research, and the Kidney Research Unit for Wales Foundation. The authors also thank Professor John Couchman of the Cell Adhesion and Matrix Research Center, Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL, USA, and Professor John Gallagher and Dr. John Deakin of the Department of Medical Oncology, Paterson Institute for Cancer Research, Manchester, UK, for their helpful comments and discussions.

Reprint requests to Robert Steadman, Ph.D., Institutes of Nephrology, University of Wales College of Medicine, Heath Park, Cardiff, CF14 4XN, Wales, United Kingdom.
E-mail: steadmanr@cf.ac.uk

APPENDIX

Abbreviations used in this article are: ATP, adenosine 5′-transferase; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylediametetraacetic acid; FCS, fetal calf serum; FGF-2, basic fibroblast growth factor; GAG, glycosaminoglycans; HS, heparan sulfate; LH, latalbumin hydrolysate; M-MLV, Maloney monkey leukemia virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NRK49F, renal fibroblasts; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PG, prostaglandins; RT-PCR, reverse transcription-polymerase chain reaction; TGF-β, transforming growth factor-β.

REFERENCES

1. Cattell V: Macrophages in acute glomerular inflammation. (editorial) *Kidney Int* 45:945–952, 1994
2. Risdon RA, Sloper JAC, de Wardener HE: Relationship between renal function and histological changes found in renal biopsy specimens from patients with persistent glomerular nephritis. *Lancet* 17:363–366, 1968
3. Bohle A, Mackensen-Haen H, Gise HV: Significance of tubulointerstitial changes in the renal cortex for the excretory function and concentration ability of the kidney: A morphometric contribution. *Am J Nephrol* 7:421–433, 1987
4. Strutz F, Müller GA: On the progression of chronic renal disease. *Nephron* 69:371–379, 1995
5. Strutz F, Müller GA: First Gottingen symposium on renal fibrosis: Prevention and progression (7-9 July 1995). *Nephrol Dial Transplant* 11:737–739, 1996
6. Strutz F, Müller GA: Interstitial pathomechanisms underlying progressive tubulointerstitial damage. *Kidney Blood Press Res* 22:71–80, 1999
7. Rodemann HP, Müller GA, Knecht A, *et al:* Fibroblasts of rabbit kidney in culture 1: Characterization and identification of cell-specific markers. *Am J Physiol* 261:F283–F291, 1991
8. Clayton A, Steadman R, Williams JD: Cells isolated from the human cortical interstitium resemble myofibroblasts and bind neutrophils in an ICAM-1-dependnet manner. *J Am Soc Nephrol* 8:604–615, 1997
9. Clayton A, Steadman R: ICAM-1 interactions in the renal interstitium: A novel activator of fibroblasts during nephritis. *Histol Histopathol* 14:861–870, 1999
10. Clayton A, Evans RA, Pettit E, *et al:* Cellular activation through the ligation of intercellular adhesion molecule-1. *J Cell Sci* 111:443–453, 1998
11. Norman JT, Fine LG: Progressive renal disease: Fibroblasts, extracellular matrix, and integrins. *Exp Nephrol* 7:167–177, 1999
12. Coutts JC, Gallagher JT: Receptors for fibroblast growth factors. *Immunol Cell Biol* 73:584–589, 1995
13. Gallagher JT: Heparan sulphate and protein recognition: Binding specificities and activation mechanisms. *Adv Exp Med Biol* 376: 125–134, 1995
14. Hildebrand A, Romaris M, Ramsmussen LM, *et al:* Interaction of the small interstitial proteoglycan biglycan, decorin and fibromodulin with transforming growth factor-β. *Biochem J* 302:527–534, 1994
15. Humes HD, Nakamura T, Cieslinski DA, *et al:* Role of proteoglycans and cytoskeleton in the effects of TGF-beta1 on renal proximal tubule cells. *Kidney Int* 43:575–584, 1993
16. Kanwar Y: Biophysiology of glomerular filtration and proteinuria of molecular origin. *Lab Invest* 51:7–21, 1984
17. Groffen AJ, Ruegg MA, Dijkman H, *et al:* Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane. *J Histochem Cytochem* 46:19–27, 1998
18. Raats CJ, Van Den Born J, Berden JH: Glomerular heparan sulfate alterations: Mechanisms and relevance for proteinuria. *Kidney Int* 57:385–400, 2000
19. van der Woude FJ, van Det NF: Heparan sulphate proteoglycans and diabetic nephropathy. *Exp Nephrol* 5:180–188, 1997
20. Bernfield M, Kokenyesi R, Kato M, *et al:* Biology of the syndecans: A family of transmembrane heparan sulfate proteoglycans. *Annu Rev Cell Biol* 8:365–393, 1992
21. Abboud HE: Growth factors in glomerulonephritis. *Kidney Int* 43:252–257, 1993
22. Bonneh-Barkay D, Shlissel M, Berman B, *et al:* Identification of glypican as a dual modulator of the biological activity of fibroblast growth factors. *J Biol Chem* 272:12415–12421, 1997
23. Ki CW, Goldberger OA, Gallo RL, *et al:* Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue-, and development-specific patterns. *Mol Biol Cell* 5:797–805, 1994
24. Rapraeger A, Krufla A, Olwin BB: Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* 252:1705–1708, 1991
25. Gallagher JT: Heparan sulfates as membrane bound receptors for the fibroblast growth factors. *Eur J Clin Biochem* 32:239–247, 1994
26. Morita H, Shinzato T, David G, *et al:* Basic fibroblast growth factor-binding domain of heparan sulfate in the human glomerulosclerosis and renal tubulointerstitial fibrosis. *Lab Invest* 71:528–535, 1994
27. Mossmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63, 1983
28. Thomas GJ, Bayliss MT, Harper K, *et al:* Glomerular mesangial cells in vitro synthesize an aggregating proteoglycan immunologically related to versican. *Biochem J* 302:49–56, 1994
29. Thomas GJ, Shrewing L, McCarthy KJ, *et al:* Rat mesangial cells *in vitro* synthesize a spectrum of proteoglycan species including

those of the basement membrane and interstitium. *Kidney Int* 48:1278–1289, 1995
30. Yanagishita M, Hascall VC: Effects of monensin on the synthesis, transport, and intracellular degradation of proteoglycans in rat ovarian granulosa cells in culture. *J Biol Chem* 260:5445–5455, 1985
31. Yanagishita M, Hascall VC: Proteoglycans synthesized by rat ovarian granusola cells in culture: Isolation, fractionation, and characterization of proteoglycans associated with the cell layer. *J Biol Chem* 259:10620–10629, 1984
32. Keller KM, Brauer PR, Keller JM: Modulation of cell surface heparan sulfate structure by growth of cells in the presence of chlorate. *Biochemistry* 28:8100–8107, 1989
33. Kojima T, Shworak NW, Rosenberg RD: Molecular cloning and expression of two distinct cDNA-encoding heparan sulfate proteoglycan core proteins from a rat endothelial cell line. *J Biol Chem* 267:4870–4877, 1992
34. Cizmeci-Smith GLE, Youkey J, Showalter LJ, *et al:* Syndecan-4 is a primary response gene induced by basic fibroblast growth factor and arterial injury in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 17:172–180, 1997
35. Roghani M, Moscatelli D: Basic fibroblast growth factor is internalized through both receptor-mediated and heparan sulfate-mediated mechanisms. *J Biol Chem* 267:22156–22162, 1992
36. Rapraeger AC, Krufka A, Olwin BB: Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* 252:1705–1708, 1991
37. Schriever C, Breithardt G, Schmidt A: Undersulfation of proteoheparan sulfate stimulates the expression of basic fibroblast growth factor and protein synthesis but suppresses replication of coronary smooth muscle cells. *Biol Chem* 378:701–706, 1997
38. Floege J, Eng E, Lindner V, *et al:* Rat glomerular mesangial cells synthesize basic fibroblast growth factor. *J Clin Invest* 90:2362–2369, 1992
39. Fernig DG, Gallagher JT: Fibroblast growth factors and their receptors: An information network controlling tissue growth, morphogenesis and repair. *Prog Growth Factor Res* 5:353–377, 1994
40. Goldfarb M: The fibroblast growth factor family. *Cell Growth Differ* 1:439–445, 1990
41. Johnson D, Williams LT: Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res* 60:1–41, 1993
42. Emoto N, Isozaki O, Ohmura E, *et al:* Degradation of cell surface heparan sulfates decreases the high affinity binding of basic FGF to endothelial cells, but not to FRTL-5 rat thyroid cells. *Thyroid* 5:455–460, 1995
43. Jayson GC, Gallagher JT: Heparin oligosaccharides: Inhibitors of the biological activity of bFGF on Caco-2 cells. *Br J Cancer* 75:9–16, 1997
44. Jayson GC, Lyon M, Paraskeva C, *et al:* Heparan sulfate undergoes specific structural changes during the progression from human colon adenoma to carcinoma in vitro. *J Biol Chem* 273:51–57, 1998
45. Pye DA, Kumar S: Endothelial and fibroblast cell-derived heparan sulphate bind with differing affinity to basic fibroblast growth factor. *Biochem Biophys Res Commun* 248:889–895, 1998
46. Brickman YG, Ford MD, Gallagher JT, *et al:* Structural modification of fibroblast growth factor-binding heparan sulfate at a determinative stage of neural development. *J Biol Chem* 273:4350–4359, 1998
47. Guimond S, Maccarana M, Olwin BB, *et al:* Activating and inhibitory heparin sequences for FGF-2 (basic FGF): Distinct requirements for FGF-1, FGF-2, and FGF-4. *J Biol Chem* 268:23906–23914, 1993
48. Carey D: Syndecans: Multifunctional cell-surface co-receptors. *Biochem J* 327:1–16, 1997
49. Pye DA, Gallagher JT: Monomer complexes of basic fibroblast growth factor and heparan sulfate oligosaccharides are the minimal functional unit for cell activation. *J Biol Chem* 274:13456–13461, 1999
50. Phillips AO, Topley N, Morrisey K, *et al:* Basic fibroblast growth factor stimulates the release of pre-formed TGF-β1 from human proximal tubular cells in the absence of *de-novo* gene transcription or mRNA translation. *Lab Invest* 76:591–600, 1997
51. Okada H, Strutz F, Danoff TM, *et al:* Possible mechanisms of renal fibrosis. *Contrib Nephrol* 118:147–154, 1996
52. Okada H, Strutz F, Danoff TM, *et al:* Possible pathogenesis of renal fibrosis. *Kidney Int* 49(Suppl 54):S37–S38, 1996
53. Muller GA, Schettler V, Muller CA, *et al:* Prevention of progression of renal fibrosis: How far are we? *Kidney Int* 49(Suppl 54):S75–S82, 1996
54. Morita H, David G, Mizutani A, *et al:* Heparan sulfate proteoglycans in the human sclerosing and scarring kidney: Changes in heparan sulfate moiety. *Contrib Nephrol* 107:174–179, 1994
55. Muller GA, Rodemann HP: Characterization of human and renal fibroblasts in health and disease. I. Immunophenotyping of cultured tubular epithelial cells and fibroblasts derived from kidneys with histologically proved interstitial fibrosis. *Am J Kidney Dis* 17:680–683, 1991
56. Rodemann HP, Müller GA: Characterisation of human renal fibroblasts in health and disease. II. In vitro growth, differentiation, and collagen synthesis of fibroblasts from kidneys with interstitial fibrosis. *Am J Kidney Dis* 17:684–686, 1991
57. Jaakkola P, Maatta A, Jalkanen M: The activation and composition of FiRE (an FGF-inducible response element) differ in a cell type- and growth factor-specific manner. *Oncogene* 17:1279–1286, 1998